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The major goal of this project is to optimize the strategies for using radioactive strontium for palliation of bone pain due to breast cancer metastases. One approach is to determine ways of selectively increasing strontium uptake at the metastatic site. Estrogens and a variety of other pharmacological agents can influence cellular uptake of calcium and and strontium. Cultures of hormone sensitive and hormone independent breast cancer cells and fetal steoblasts and osteoclasts are being examined for their ability to accumulate strontium.Therefore we have standardized the method for estimating strontium in cell extracts using atomic absorption spectrometry. Bisphosphonates are useful in the treatment of osteoporosis and bone metastases. Bisphosphonates sequester calcium and strontium. Bisphosphonates are charged molecules that do not easily cross hydrophobic cell membranes. The nature of the counter ion to the anionic phosphonate was found to have an effect on the cellular uptake of bisphosphonate such as etidronate. Compounds such as spermidine enhanced the effect of etidronate, presumably by increasing cellular uptake.

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INTRODUCTION

In the first year of this project we have concentrated mainly on establishing monolayer cultures of human fetal osteoblasts, breast cancer cells and murine osteoclasts. We have also established the conditions of low calcium levels for tissue culture experiments. Experiments are in progress for estimating calcium and strontium in cells. We have just initiated experiments for covalent modification of bisphosphonates. Thus tasks 1, 3, 6 and 7 have been initiated. We have not submitted any manuscripts for publication during the period covered by this report, but one manuscript is under preparation for submission to a peer reviewed journal.

BODY

BACKGROUND

More than 50% of patients with primary breast cancer will eventually develop bone metastases and 75% of patients with breast carcinoma were found to be with bone metastases at autopsy (1,2). In spite of skeletal metastases, breast cancer patients can survive for long periods (5 year survival is about 20%), but these persons will have much pain and suffering due to cancer mediated bone destruction (2). In addition to bone pain, these individuals may develop bone fractures, spinal cord compression (which can cause paralysis) and hypercalcemia of malignancy (HCM) (2-4). Breast neoplasms have a strong tendency to metastasize to the bone. For this to occur, both the tumor cell and host cells at the site of metastasis interact significantly and selectively (5-7). Once the cancer cell reaches the bone surface, the proteolytic enzymes of the neoplastic cell can facilitate the break down of the stroma and pave the way for metastasis to become entrenched. Another possibility is for the cancer cell to recruit and promote the proliferation of osteoblasts (bone forming cells) and new bone formation. Soluble growth factors from the cancer cell enable new bone to be formed around the neoplastic cell. Alternatively the growth factors produced by the migrating tumor cell could stimulate the proliferation of osteoclasts which are involved in bone destruction. The breast carcinoma cells can also destroy the bone. Destruction of the bone by the metastatic process can lead to hypercalcemia and associated problems. Certain breast tumors produce parathyroid hormone which promotes bone resorption. In particular, parathyroid hormone stimulates osteoclasts which degrade the bone. It appears that parathyroid hormone stimulates the growth of osteoclasts, and enhances parathyroid hormone production by the bone compared to normal tissues and tumor (7,8). Other factors known to increase osteoclast mediated bone resorption are interleukin 1 (9), interleukin 8 (10), interleukin 6 (11) and cathepsin K (12).

Preosteoblasts are differentiating intermitotic cells derived from vegetative intermitotic mesenchymal stem cell. The preosteoblasts differentiate further to yield mature osteoblasts that are involved in bone matrix synthesis. Preosteoclasts are also of the

differentiating intermitotic type that are derived from hematopoietic stem cell. Preosteoclasts differentiate further into mature multinucleated osteoclasts that are involved in bone resorption. Cytokines such as TGF-β, macrophage-colony stimulating factor (M-CSF), IL-6. TNF-α, and osteoprotogerin (OPG)/osteoprotogerin ligand (OPG-L) from a variety of sources including mature osteoblasts are involved in promoting proliferation and differentiation of preosteoclasts to mature osteoclasts (13).
Estrogen deficiency is associated with enhanced bone osteoclast formation and bone loss. Correction of estrogen deficiency prevents bone loss. Estrogen action is mediated through cytokine production. Bisphosphonates also protect against bone loss. There is evidence to suggest that both estrogen and bisphosphonates can have direct effects on osteoblasts and osteoclasts (13-15). There are examples of bisphosphonates covalently linked to estrogens and anti-estrogens (16,17). The anti-estrogen, tamoxifen and selective estrogen response modifiers protect against osteoclast mediated bone resorption (18,19). The effects of conjugates of bisphosphonates with estrogens and anti-estrogens on osteoblasts and osteoclasts are not known. Investigation of such conjugates may help identify compounds that protect against bone lysis caused by breast cancer metastases.

The radioisotope Strontium-89 (Sr-89) has a physical half life of 50.5 days. It is a pure beta emitter (undergoes beta minus decay with a beta emission of 1.463 MeV). The maximum range of the beta emission is about 8 mm. Strontium is a calcium mimic and a bone seeking mineral. When in the bone the radiation from this isotope is mainly to the cortical and trabecular bone, with less to bone marrow and minimal dose to adjacent soft tissues. Strontium-89 is usually given at a dose of 4 mCi (or 40-60μCi/kg body weight [1.5MBq/kg] is used as a single injection. About 65% of the patients gain relief from pain. The success of Sr-89 treatment depends on the ability of the radioisotope to infiltrate into the osteoblasts and osteoclasts, Once the radioisxzotope is taken up by the cells, the beta emission can damage the cell. If the isotope is retained long enough then the accumulated damage can be lethal to bone cells. Strontium-89 has been used for palliative treatment of bone metastases from breast and other cancers (20-24). Strontium-89 is sold under the trade name metastron. We have used metastron for treating bone pain in about 41 patients (25). There is considerable variation in treatment response with respect to pain relief. There may be several reasons for this. For this treatment to be effective, radioactive strontium has to be taken up in sufficient amounts by the intended target (osteoblasts and or osteoclasts). Sr-89 does not exert an appreciable lethal effect towards tumor cells. Since strontium is a calcium analog, the uptake of strontium may depend on a variety of factors including systemic calcium levels. If the systemic calcium levels are high, cellular uptake of strontium may be poor because calcium may competitively inhibit the uptake of strontium.

Bisphosphonates are useful in the treatment of bony metastases, especially those traceable to an osteoclast etiology (26-34, 35-37). Bisphosphonates and estrogen metabolites appear to have a direct effect on bone cells (38,39). Bisphosphonates also appear to have antineoplastic effect by inducing apoptosis of tumor cells *in vivo* and *in vitro* (40). Osteoprotegerin (OPG) is a potent inhibitor of osteoclast formation and activity. OPG is a decoy receptor which neutralizes OPG-ligand (OPG-L) which is the ultimate effector of osteoclastogenesis (41).

HYPOTHESIS/RATIONALE/PURPOSE

The purpose is to improve the use of Sr-89 and bisphosphonates for treating bone metastases from breast cancer. The rationale is as follows: Nearly 50% of breast cancer patients develop bone metastases due to osteotropic nature of the primary breast cancer cells. Breast cancer cells, osteoblasts and osteoclasts have some growth factors in common. Thus the breast cancer cells promote the growth of either or both types of cells. The osteoblsts surround the cancer cell which has adhered to the bone matrix and starts depositing new bone which ultimtely leads to bony metastasis. In contrast, the osteoclasts utilize the growth factors differently. They destroy the bone by resorption which results in hypercalcemia.

Sr-89 is usually satisfactory for the treatment of lesions caused by osteoblasts. Osteoblastic activity attracts breast cancer cells and utilizes calcium for making new bone. Strontium is an analog of calcium. Sr-89 is easily taken up instead of calcium by the active osteoblasts. Once inside the osteoblast, the beta emission from the radionuclide inactivates the osteoblast. This can lead to some relief from pain, If the lesion is caused by osteoclast, which does not consume calcium or strontium ions avidly, Sr-89 is not quite so effective against osteoclasts. Bisphosphonates are pyrophosphate analogs which have a rather high affinity for bone. Although bisphosphonates such as pamidronate are useful in the treatment of bony metastases, it is important to know if the high levels of hypercalcemia have to be decreased in order to achieve effective treatment with pamidronate or Sr-89. Since bisphosphonates and Sr-89 complement each other, addition of a bisphosphonate can enhance the efficacy of Sr-89 and vice versa. Such logic can be extended to arrive at other combinations based on our knowledge of the mechanisms involved.

Hypothesis 1: Calcium concentration in the system affects strontium uptake by the cells. For improved use of Sr-89, strategies for decreasing cellular calcium pools are necessary. Gallium nitrate or a bisphosphonate can be used for lowering the calcium levels in cells.

Hypothesis 2: Antiestrogens and estrogens can modify the effects of bisphosphonates on osteoblasts and osteoclasts. Bisphosphonates linked covalently to anti estrogen and estrogen moieties will have greater affinity for bone cells and may be more active.

Hypothesis 3: Hormones and hormone antagonists can be combined for modulating the effects bisphosphonates on bone cells.

Specific Aims:

1. Treat osteoblast and osteoclast-like cells in culture with graded concentrations of strontium in the presence of different amounts of calcium and measure the kinetics of uptake and retention of strontium by cells. Atomic absorption spectroscopy will be used for estimating strontium concentration.

2. Test the influence of the bisphosphonates pamidronate and its more potent analog zolendronate on the uptake of strontium by the osteoblast cell lines in culture. It is not known if bisphosphonates alter the bioavailability of strontium ions.

3. Synthesize bisphosphonates conjugated to estrogen and anti-estrogen moieties and test their effects on the viability of breast cancer cell lines and human fetal osteoblasts in culture. Cell viability will be assessed on the basis of apoptosis assays and clonogenicity measurements where feasible.

4. Determine if gallium nitrate, calcium channel blockers, nifedipine and verapamil and the antiestrogens tamoxifen and raloxifene alter strontium uptake by human fetal osteoblasts.

Grown as monolayers and as multicell spheroids.

******Both estrogen receptor positive and receptor negative human fetal osteoblasts and breast cancer cells will be utilized in our experiments. Estrogen receptor positive human fetal osteoblast (hFOB/ER9) and estrogen receptor negative human fetal osteoblasts (hFOB1.19) are to be studied. Estrogen responsive MCF-7 and estrogen independent MDA-MB231 are among several breast cancer cells in our laboratory.

STATEMENT OF WORK

Task 1.	Establish osteoclast and osteoblast cultures (months 1 through 6). Standardize assays for strontium and calcium using atomic absorption spectrophotometry (months 1 through 3). Determine the baseline values of strontium and calcium in cultures grown in defined media. Evaluate the effect of calcium levels in the medium on strontium uptake by hFOB1.19 and hFOB ER/9. Compare the results obtained using serum-free and serum supplemented culture media. Test the effects of gallium nitrate and calcium channel blockers verapamil and nifedipine on strontium uptake and retention (months 1 through 15).
Task 2.	Synthesize bisphosphonates conjugated to estrogenic and antiestrogenic moieties (months 1 through 18).
Task 3.	Test the effects of bisphosphonates (including pamidronate, zolendronate and those synthesized in task 2) on strontium uptake by osteoblasts (months 6 through 30).
Task 4.	Determine the influence of gallium nitrate on the uptake of strontium by the afore-mentioned cell lines (months 2 through 12).
Task 5.	Measure the uptake and retention of strontium by multicellular spheroids of osteoblasts in the presence and absence of agents which affect calcium homeostasis (months 6 through 30).

Task 6. Evaluate the differences, if any, in the uptake kinetics of strontium and calcium in the different cell lines. Determine any correlations that may exist among the cellular levels of calcium and strontium on the cytotoxicity of bisphosphonate. Utilize isobologram analysis to reveal any synergistic or antagonistic interactions between bisphosphonates and strontium and/or calcium (throughout the 36 month project period).

Task 7. Assays for strontium, calcium and clonogenicity assays for cell viability and apoptosis will be carried out throughout the 36 month project.

Methods:

Cell lines: Estrogen responsive MCF-7 and estrogen independent MCF-7MDR clone 10.3 human breast cancer cell lines were maintained as monolayer cultures growing in RPMI-1640 medium supplemented with 10% fetal bovine serum, glutamine, pyruvate, insulin, penicillin and streptomycin. MCF-7 cells were also grown as multicellular spheroids.

Culturing hFOB 1.19 and hFOB/ER cell lines. These osteoblast cells (42,43) were obtained from Dr. Thomas Spelsberg of Mayo Clinic, Rochester. (The letter confirming this is enclosed in the appendix section). The following is their description of the cells along with the recommended procedure for the growth and maintenance of these cells.

The hFOB 1.19 cells were cultured as monolayers at 34° C in DMEM-F12 medium supplemented with 10% fetal bovine serum and 300μg/ml geneticin, and induced to differentiate more fully either by culturing past confluence or by culturing at 39° C, as described by Harris SA et al, Bone Miner Res 10:178-186, 1995 (42).

The hFOB/ER9 cell lines were derived from the hFOB 1.19 cells were also cultured at 34° C in DMEM:F12 (1:1), but supplemented with 10% charcoal-stripped FBS and either geneticin at 300μg/ml or hygromycin B at 100μg /ml.

Strontium uptake studies: Monolayer cultures were trypsinized to yield single cell suspensions, which were incubated in the presence of graded concentrations of the different strontium chloride for different durations. The incubation mixture was layered over a denser than water mixture of silicone oil in a centrifuge tube. The cells are centrifuged to the bottom of the tube and the cell pellet at the bottom was harvested and extracted with hot hydrochloric acid and lanthanum oxide for determination of strontium

by atomic absorption spectroscopy. We have the necessary calibration standards for strontium determination.

MTT Assay for Cell Viability:

The cells were treated with graded concentrations of etidronate for different treatment periods of incubation (1,3, 6 and 12 hrs). Mitochondial dehydrogenase levels, which are correlated to cell viability, were determined by the enzyme mediated cleaving of the tetrazolium salt ring of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide to yield purple formazan crystals. These crystals were dissolved in isopropanol, and the absorption at 560 nm was determined spectrophotometrically. Appropriate control experiments were run to determine spectrophotometric background and absorption due to reagent blanks.

Apopain Assay:

Apopain/Caspase 3 is derived from the proenzyme CPP32 at the onset of apoptosis and play a pivotal role in programmed cell death. This assay indicates apopain activity associated with apoptosis. The FluorAce apopain assay kit was used (Bio-Rad, Catalog number 170-3130).

Flow Cytometry Analysis for Cell Cycle and Apoptosis:

The effect of etidronate treatment on cell cycle and apoptosis was analyzed using flow cytometry assay. The cells were trypsinzed and washed twice with PBS after treatment. The suspended cells were fixed with 80% ethanol for 30 min on ice, and then centrifuged for 5 min at 1500rpm. The fixed cells were washed again with PBS and the supernatant was removed. The cells were stained at 4° C in the dark with 1 ml of propidium iodide (PI) solution and/or fluorescein-conjugated annexin V (apoptotic marker), and then stored at 4°C until analysis. The cell cycle distribution was analyzed by FACS caliber flow cytometry (Becton Dickson, San Jose, CA). Ten thousand cells were analyzed per sample. PI solution contained 100 Units/ml or 50 μg/ml RNase A and 50 μg/ml PI in PBS.

Similarly the effect of different concentrations of strontium chloride on cell cycle was analyzed using flow cytometry.

KEY RESEARCH ACCOMPLISHMENTS

- Monolayer cultures of the cell lines needed for our research have been established, but only MCF-7 human breast cancer cells have been grown as multicell spheroids.

- Assays for cell viability have been standardized and utilized for evaluating the cytotoxicity of etidronate and analogs towards the different cell lines in culture.

- Assays for strontium and calcium have been standardized.

- The importance of counter ion for cellular uptake of bisphosphonate has been demonstrated.

- Toxicity of etidronate towards osteoclast and breast cancer cells was demonstrated.

- Flow cytometry assays for cell cycle analysis and estimation of apoptosis have been standardized and applied to study the effect of etidronate towards breast cancer cells.

REPORTABLE OUTCOMES

None for the period covered by this report. One manuscript is in preparation for submission to *Pharmacological Research.*

CONCLUSIONS

Cells detach from the substratum when calcium is depleted from the culture medium. The bisphosphonate etidronate is toxic to breast cancer cells and osteoclasts. Combining etidronate with spermidine increases the cytotoxicity of etidronate.Etidronate influences the uptake of strontium by cells

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